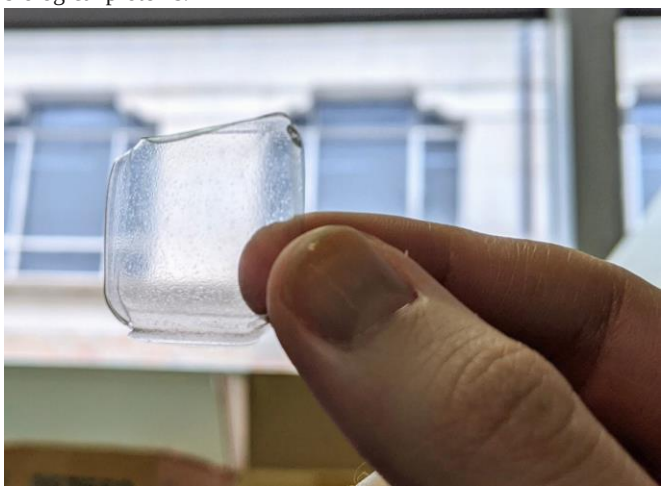
subviral particulates at day 30. Stability profiles of the same preparation stored at 4 °C for 30 days were not statistically different from vector stored in film at room temperature at the same time point. Films taken from room temperature storage and placed at 40 °C with 20% relative humidity for 3 days demonstrated a loss of less than 5% of infectious titer. Initial mechanistic studies suggest that elements of the film matrix directly bind to capsid proteins to stabilize them and shield them against environmental stressors. *In vivo* studies in mice demonstrated that vector biodistribution and transgene expression profiles of vector dried within the film matrix were similar to those of vector stored frozen in the commerical formulation. Taken together, these results suggest that storage of AAV in our novel matrix facilitates easy transport of vector to remote sites without compromising in vivo performance. Companion studies using enveloped and non-enveloped viruses (Ad, influenza) further suggest that this approach could be a universal technology for room temperature storage of vaccines, gene therapy vectors, and biological proteins.



167. Exposing the Content of Different AAV Fractions after Ultracentrifugation

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Recent developments in the biomedical fields have enabled that viruses can also be used to help us fight against numerous diseases. Even though the production of recombinant viral vectors began 40 year ago, the established technologies still haven't reached the optimal level.

Different product and process related impurities affect the efficiency of the production. To improve the overall production process, advanced analytical techniques are needed to accurately follow presence of desired product and possible impurities in various production steps. A variety of methods have been developed and utilised to characterize viral vectors, but only a limited number of comparative studies have been done to demonstrate correlations between them. Non-related analytical techniques usually also produce non-comparable results. We have used different analytical techniques (Quantitative realtime PCR (qPCR), digital droplet PCR (ddPCR), high throughput sequencing (HTS), enzyme-linked immunosorbent assay (ELISA) and transmission electron microscopy (TEM)) to thoroughly characterize four fractions of AAVs (empty, intermediate, full and heavy particles) coming from CsCl gradient purification. The aim of the study was to compare those different fractions in terms of vector and impurities content and at the same time evaluate the correlations between different analytical methods. The results have shown that the fractions were relatively similar in terms of relative content of vectors and impurities, with exception of heavy fraction. There was no clear correlation between the results of different methods that would hold for all of the fractions. Nevertheless, the results offered an interesting insight into the presence of impurities, vector genomes and viral particles in different fractions. Those results will be presented and discussed in view of content of partially filled vectors, comparability of analytical methods and importance for creating a wider picture of the virus vector sample.

168. Optimization of Affinity Purification for Adeno-Associated Viral Vectors

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Purification of recombinant AAV (rAAV) via affinity chromatography with Poros™ CaptureSelect™ (PCS) AAVX, AAV8 or AAV9 affinity resins achieves good purity and high vector concentration with minimal vector loss. Additionally, this purification process is highly scalable. Despite the advantages of affinity purification, we and others have observed that significant host cell proteins (HCPs) and other impurities such as endotoxins are co-eluted with rAAV. The goal of this study was to optimize the washing step during affinity chromatography to remove contaminants and increase rAAV purity. In this study, we systematically evaluated a variety of washing reagents including 1M NaCl, 0.2 M $MgCl_2$, low pH (pH4 and pH5), 0.5 M arginine, 0.1% Tween 20 and 1-2% OTG (octyl β-D-1-thioglucopyranoside) for their ability to improve rAAV8-EGFP affinity purification using PCS AAVX. In these experiments purity was evaluated by Coomassie blue stain of SDS-PAGE gels, titer by the CyQuant method, and endotoxin by the

Limulus Amebocyte Lysate (LAL) method. A large batch of rAAV8EGFP was generated and used as the starting material for all rAAV8 affinity purifications. Gradients of wash buffers were applied in some purifications to identify the best concentration of the reagent. In these studies, we observed that some of the wash buffers tested resulted in the loss of rAAV8 by capsid protein immunoblot including 0.1% Tween 20, 50 mM Citrate buffer (pH 4.0) and 0.5 M arginine. These findings highlight the challenge of identifying washing buffers that can remove impurities while retaining the association of the rAAV and the affinity ligand. rAAV8 vector was not washed off by 1M NaCl, 0.2 M $MgCl_2$, 50 mM Citrate buffer (pH 5.0), or 2% OTG as determined by immunoblot.

Compared to PBS wash alone, these reagents reduced the amount of contaminating proteins and endotoxins (about 66-100%) from the vector preparation. Among the reagents evaluated, 2% OTG resulted in the greatest reduction in contaminating proteins and endotoxins (<LoD) compared to PBS wash alone. Using this protocol we achieved yields approximately 88% of PBS wash alone suggesting OTG wash results in minimal vector loss. Our preliminary data also showed that OTG wash can be used to purify rAAV1 (1% OTG wash), rAAV2 (1% OTG wash), and rAAV5 (2% OTG wash) with PCS AAVX resin. A 2% OTG can also be used to successfully purify rAAV9 with PCS AAV9 resin. We observed approximately half of rAAV9 was lost after 2% OTG wash using PCS AAVX resin suggesting differences in how rAAV9 interacts with the two resins. Currently, other serotypes are being tested with OTG wash. Our group previously published an optimized rAAV production protocol in suspension HEK 293T cells with tripletransfection (Zhao, et al. Mol Ther Clin Dev & Methods, 2020). When this OTG wash was combined with a modified protocol that eliminates sodium butyrate, we were able to produce about 5-6×10^{14} VG of purified rAAV8-Ef1α-EGFP from one liter of suspension HEK 293T cell culture. The yield achieved via this suspension HEK 293T cell platform and our affinity purification scheme offers high scalability and a comparable yield to *sf9* cells (Kurasawa et al. Mol Ther Clin Dev & Methods, 2020) making it an appealing option for rAAV generation.

169. Characterization of rAAV Key Quality Attributes Generated from a Highly Optimized, Hela 3.0 Producer Cell Line (PCL) Production Platform

Nicholas Richards[1], Caroline Adams[2], Kevin Larpenteur[2], Justin Tedeschi[2], Samuel Wadsworth[2], K. Reed Clark[2], Matthew Fuller[2]

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A key challenge facing the gene therapy field today is ensuring that manufacturing capabilities surpass current standards to ensure accessibility and affordability for all eligible patients, as well as facilitating treatments for diseases that require higher doses for efficacy, such as Duchenne muscular dystrophy. To address these needs, we have developed a HeLa-based rAAV manufacturing platform that has demonstrated scalable 2000L production in an industrial setting to support ongoing Phase I/II clinical trials. Recently, we have further optimized our HeLa Producer Cell Line (PCL) platform to HeLa 3.0 by genetically modifying existing, highly productive monoclonal PCLs. Utilizing an RNA-seq directed screening method, we identified specific genes that modulate rAAV production and knocked out those genes *via* CRISPR/Cas9-mediated genome editing, which increased productivity up to 5 fold. While titer improvements are important, ensuring the quality and fidelity of both the production platform and manufactured vector is vital. To this end, two HeLa 3.0 producer cell lines, developed for separate clinical programs, were interrogated to characterize attributes previously identified as essential for generation of a robust, stable cell line including rAAV genome amplification, integration site analysis, kinetic analysis of rAAV production and overall fitness. In addition, viral products generated from both HeLa 3.0 PCLs were analyzed for key quality attributes, such as genome integrity,

capsid protein ratio, and empty: full ratio to establish comparability with existing, efficacious vectors. Importantly, the newly optimized HeLa 3.0 production system demonstrated the robustness required to facilitate production of high quality rAAV vector product.

170. AAVX Resin Binding Site Identification via Library Screening Analysis on Novel AAV Vectors

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A major effort by AAV-based gene therapy manufacturers is focused on improving yield and reducing COGs independent of AAV serotype to enable the potential treatment of rare and non-rare diseases with a one-time dose of gene therapy. Key among these efforts is improved affinity capture. Affinity capture is a scalable method that effectively removes host impurities from AAV vector preparations. As with any receptor/ligand complex, the stringency of capture of the AAV particle is a function of the dissociation constant (Kd) of the interaction. Identifying and understanding the specific chemistries of the interaction may allow engineering and modulation of the Kd. Similar to AVB, AAVX is an affinity resin that utilizes a camelid antibody to capture AAV vector particles. Camelid antibodies are single chain and exhibit extended CDRs, making them ideal for reaching confined epitopes and conjugation to porous substrates. The development of the resin by ThermoFisher has been recognized as a major improvement to downstream processes. AAVX resin can bind most tested serotypes with an affinity high enough to withstand the stringent wash conditions required for therapeutic development. Here, we investigate the potential AAV epitope responsible for binding to AAVX resin. The use of AAV vector libraries to address manufacturability concerns has not been widely reported. The rich datasets produced using diverse yet informatically manageable vector libraries engineered via ancestral sequence reconstruction provide an opportunity to answer questions of manufacturability at multiple process steps. Exemplifying this utility, we have preliminarily identified the region of the AAV vector where the AAVX epitope resides. Processing the Anc80 vector library via AVB and AAVX affinity resins revealed differential efficiency of vector capture. By informatic analysis, the single residue responsible for this observation was identified. Interestingly, this binding site is located within the 3-fold axis, which is distinct from the AVB binding site near the 5-fold axis. Further studies to

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definitively identify critical primaryto-tertiary elements of the AAVX epitope are ongoing.

171. Analysis of Gene Therapy Products by Charge Detection Mass Spectrometry

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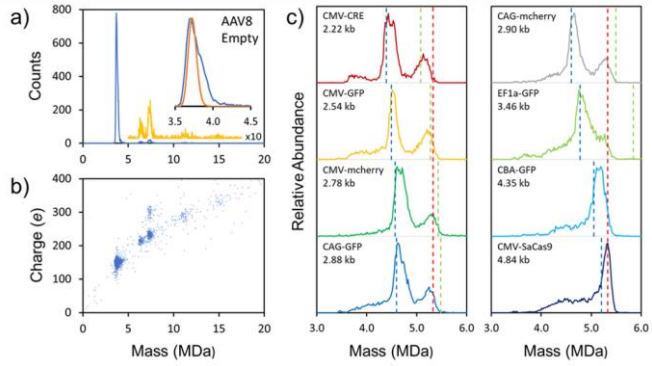
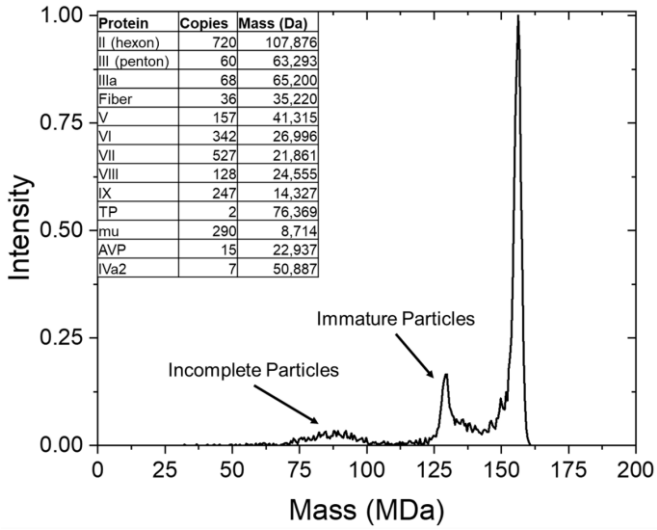
New analytical tools in cell and gene therapy will improve patient outcomes. In general, commercial mass spectrometers have an effective upper mass limit around a megadalton (MDa), a limit that arises because of the loss of charge state resolution in the mass-tocharge (*m*/*z*) spectrum. Cell and gene therapy platforms fall beyond this range, but a relatively new technique called charge detection mass spectrometry (CDMS) can circumvent this limitation and bring the precision of mass spectrometry to cell and gene therapy. CDMS simultaneously measures the *m/z* and charge (*z)* of individual particles allowing for a direct calculation of mass. This extends accurate mass measurements into the MDa to gigadalton (GDa) regime. Accurate mass measurements obtained from CDMS can be used to assess a range of critical quality attributes for gene therapy products. This work will highlight how CDMS has been demonstrated for viral vector platforms and genomic material. Adeno-associated virus (AAV) vectors have a mass in the 3-5 MDa range depending on the packaging of genomic material. Direct mass measurement allows for the packaging to be assessed and the gene of interest to be identified for intact capsids. We show that the differences between the masses of empty particles and particles with the genome of interest (GOI) are correlated with the expected genome mass. In addition, CDMS provides a robust orthogonal technique to quantify empty, partial, and full particles as well as aggregates that form through both capsid degradation and capsid adherence. Adenovirus vectors have a well-established use in cancer treatments and hold great promise in vaccine research. Here we show the first high resolution mass measurement for a virus with a mass over 100 MDa. Using CDMS, we correlate the measured mass for a range of packaged genome lengths to the amount of co-packaged counterions and proteins important for genome stability. In addition, we use the accurate charge measurement to assess structure and identify incomplete, defective, and empty particles of adenovirus. Due to the importance of genomic material in gene therapies, we show how CDMS can assess the purity and mass of plasmids. Building off the mass measurements of plasmids we also able to directly detect and measure the genomic material from disassembled AAV and adenovirus capsids. In the case of AAV both ssDNA and dsDNA corresponding to the GOI were detected and used to identify the purity of the packaged contents. All spectra and results showed above use a single CDMS platform that is robust and highly sensitive. A few benchmarks include: AAV analysis low titer samples (1e10 vp/mL) in heterogeneous matrices, mass measurements with low volumes (10-20 μL), and analysis times of less than 1 hour.



(Figure 1)



(Figure 2)

172. Use of SPTFF in Continuous Downstream Manufacturing of Adeno-Associate Viruses

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In recent years, pre-clinical and clinical development in the gene therapy industry has been rapidly growing. To meet the industry's requirement for large quantities of GMP-compliant therapeutic viral vector, there's a need for high-efficiency equipment and consumables. A typical downstream process for AAV manufacturing involves a combination of unit operations including clarification of crude harvest, chromatography, concentration, diafiltration and sterile filtration. A few challenges in AAV processing include processingtime and shear sensitivity of the product, and safety concerns of the product. Adding TFF membranes into a process can reduce working volumes, and application of single-use consumables can mitigate safety concerns. Replacing traditional recirculating TFF with newer Single-Pass TFF (SPTFF) technologies has the potential to reduce shear exposure, reduce processing time by integrating with unit operations before and/or after, and improve process yields. In this work we implemented Palls innovative Cadence SPTFF technology for in-line concentration to optimize an AAV downstream process. An SPTFF device was connected to an upstream depth filtration assembly with a small break tank. The post-

SPTFF-concentrated viral vector stream was continuously pumped through a sterile filter. This work demonstrates the use of an integrated, continuous SPTFF operation in an AAV process that achieves 40% reduction in processing time while maintaining a 96% Yield.

Immunotherapy and Vaccines

Immunotherapy and Vaccines

173. A Humanized EBV Mouse Model to Evaluate the Safety/Impact of Human T_{reg} Cell Therapy on Antiviral Immune Responses

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Regulatory T cells (Treg) suppress immune cell activation and thus have key roles in preventing excessive immune responses and autoimmunity. Adoptive cell therapy with Treg is currently being tested clinically to provide tolerance for graft vs. host disease, organ transplant and autoimmune diseases. Due to potential limitations of Treg frequency, stability and ex vivo expansion, we have utilized gene editing to enforce FOXP3 expression in peripheral blood $CD4^+$ cells, driving them to adopt Treg-like phenotypes and functions. We refer to these cells as engineered Tregs (Eng Tregs). While Eng Tregs show robust suppression of pathological immune responses in mouse models, it is theoretically possible that adoptively transferred Treg could have undesirable off-target effects such as suppressing immune responses against pathogens, either during novel exposures or the reactivation of latent viruses such as Epstein-Barr virus (EBV). While most humans (95%) will have been infected with EBV by adulthood, infection is usually well-controlled by the immune system. However, a reservoir of latently infected B cells persists for decades, kept in check by immunological surveillance; the latent EBV can become reactivated when the immune system is suppressed. The goal of our study was to evaluate the safety/impact of engineered Treg (Eng Tregs) on viral responses. Since EBV does not infect mouse cells, we tested this in the following humanized mouse model. From a G-CSF-mobilized human donor, we purified autologous $CD34^+$ peripheral blood stem cells (PBSC), $CD4^+$ T cells, and thymic Treg (tTreg). The $CD34^+$ PBSCs were transplanted into busulfan-conditioned NOD-*scid*-$IL2Rg^{NULL}$ (NSG) mice. After stable engraftment (12-13-weeks post-transplant), humanized NSG were infected with EBV, then subsequently treated with either autologous tTreg or Eng Tregs ($CD4^+$ T cells edited to stably express FOXP3 utilizing HDR-based CRISPR/Cas9 targeted integration of FOXP3 cDNA with an MND promoter into *FOXP3)*. Eight weeks later, we quantified circulating EBV loads and the immunophenotype of human lymphocytes in the spleen, blood, and bone marrow. These experiments included control mice that were treated with T celldepleting antibodies in place of Treg; these mice rapidly developed B cell proliferative disease associated with EBV infection/re-activation and had high EBV loads vs. mice receiving EBV alone. This control demonstrated the role of the human $CD4^+$ and $CD8^+$ T cells in keeping the EBV infection in check in this model. Importantly, the viral loads, % human B cell and % activated human $CD8^+$ T cells in response to EBV infection were equivalent between mice that received Eng Tregs, tTreg, or EBV alone. Further, we were able to detect $LNGFR^+$ $FOXP3^+$ cells in the Eng Tregs cohort at his time point, suggesting the stability of Eng Tregs in this pro-inflammatory setting. These combined data demonstrate that Eng Tregs do not impact the immune responses required to control EBV infection in this EBV-infected humanized mouse model; demonstrating an effective in vivo system to evaluate whether human Treg-based cell therapies can preserve desired antiviral immune responses.

Immunotherapy and Vaccines

174. Candidate Selection in BALB/c Mice towards a Single Dose AAV-Based COVID19 Prophylactic Vaccine

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Ruchi Chauhan, Allison Cucalon, Cheikh Diop, Maya Kim, Abigail Sheridan, Luk H. Vandenberghe
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The public health crisis of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARSCoV-2) exceeds 100 million cases worldwide. While several protective vaccines are becoming available, several challenges to attain levels of population immunity remain. We hypothesized that, based on prior developments, an AAV-based preventative vaccine could address some of the biological and logistical limitations to an effective vaccination campaign at a global scale, importantly for example single dose efficacy. In the present study, we designed 9 AAV2/rh32.33 vector based COVID-19 genetic vaccine candidates (AAVCOVID) expressing the wild type full length spike (S) protein, stabilized S derivatives and several soluble S-based antigen, namely S ectodomain, S1 and receptor binding domain (RBD), the latter both in ssAAV and scAAV context. Wuhan S and D614G variant strains were used as the basis for antigen design. AAVCOVID candidates were evaluated for immunogenicity following single dose intramuscular (IM) injection in BALB/c mice at either 10^{10} gc/mouse or 10^{11} gc/ mouse for a period of 3 months post injection. From these studies, a candidate selection was performed for further development. Results demonstrate variable levels, yet overall rapid and robust antigen specific binding and neutralizing antibody responses. Interestingly, serum antibody isotype profiles differed qualitatively between membrane anchored antigens and soluble antigens, suggesting a more pronounce Th2 phenotype for the soluble antigen vaccine candidates. However, all candidates induced robust IFN-γ, but not IL4, T cell response by ELISpot assay at a 3-month time point, indicative of a Th1 biased response for all AAVCOVID candidates. For the duration of the immune response, the serum antibody titer reached the peak at month 2 and started to decrease at month 3. Remarkably, T cell responses in the low dose group mice were equivalent to modestly higher than those in high

dose group. Taken together, these results indicate that the AAVCOVID platform induces high level neutralizing responses that are complemented with robust T-cell immunogenicity. Based on these data and other strategic parameters, AAVCOVID-1 (membrane anchored pre-fusion stable S) and AAVCOVID-3 (secreted S1) progressed to further studies toward consideration of one candidate for clinical development.

175. AAV Specific CAR Regulatory T Cells Mitigate Immune Responses against AAV Gene Therapy

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Despite the success of AAV vectors as powerful gene therapy tools, the immune responses to AAV capsids have limited their therapeutic applications. As observed in clinical trials, systemic delivery of AAV leads to capsid specific immune responses and clearance of the transgene by CD8 T cells while intramuscular delivery of AAV, leads to long-term transgene expression despite an immune response. Differences in transgene expression is thought to be due to T regulatory cell (Treg) infiltration in muscles creating tolerance to AAV capsids. Hence, we examined the ability of Tregs to induce local and specific immunosuppression rather than steroid mediated non-specific immune suppression. We previously generated AAV specific chimeric antigen receptor T cells (AAV-CAR), and AAV specific regulatory T cells (CAR-Treg) which recognize major AAV capsid variants. AAVCARs are cytotoxic to AAV infected target cells and this killing is suppressed by AAV-CAR-Tregs. In murine models, AAV-CARs cleared AAV infected cells. Herein, we confirmed the cytotoxicity of AAVCARs and the suppressive ability of AAV-CAR-Tregs against the AAV capsid variants- AAV1, AAV2, AAV3b, AAV 5, AAV6, AAV8, AAV9 and rh32.33. Further, AAV-CAR-Tregs inhibited the proliferation and interferon gamma (IFN-γ) production of AAV-CAR T cells when stimulated with AAV capsid. Next, we utilized AAV-rh32.33 capsid to elicit a robust immune response in mice. Animals were injected with AAV-rh32.33 expressing alpha 1 anti-trypsin (AAT) followed by intravenous delivery of AAV-CAR-Tregs, non-specific expanded T-regs, or saline. AAT expression was stable for 25 weeks in both Treg and AAV-CAR-Treg groups, despite high level of anti-capsid antibody with no detectable expression 3 weeks post AAV injection in the saline group. Histology of injected muscles revealed detectable AAT staining in AAV-CAR-Treg, or non-specific Tregs groups and not in the untreated group. Tregs can induce tolerance in an antigen independent pathway via the bystander effect, where antigen activated Tregs create local suppressive environment which can suppress other antigens. To determine if AAV-CAR-Tregs can function similar to endogenous Tregs, we tested if they AAV capsid specific AAV-CAR-Tregs could block immune responses to transgene. We designed a cytotoxicity assay with mixed antigens of CAR T cells and CAR-Tregs which revealed that AAV-CAR-Tregs could suppress CAR T cells specific to other antigens. To test this principle in vivo, we delivered AAV1 expressing the immunogenic OVA (AAV-OVA) transgene followed by delivery of either AAV-CAR-Tregs, expanded non-specific T-regs, or saline. Stable transgene expression was observed in the animals receiving AAV-CAR-Tregs, or non-specific T-regs, and was reduced 2 weeks after AAV-OVA in the saline group. Interestingly, all groups produced anti-OVA antibodies despite treatment. Moreover, histology of injected muscles revealed severe focal, and diffuse myositis in untreated animals, with significantly reduced inflammation observed in the muscles of the animals treated with AAV-CAR-Tregs, despite a robust OVA expression. Together, this data demonstrates that AAV-CAR-Tregs are powerful tools to model the cellular immune response against AAV capsid. Therapeutically, AAV-CAR-Tregs potentially can modulate immune responses directed against AAV capsids and immunogenic transgenes without the detriments of systemic immunosuppression.

176. Engineered Protein M Analogs Enhance the Ability to Suppress Vector Neutralizing Antibodies and Generate a Window for Successful Gene Delivery

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Patient derived neutralizing antibodies (NAbs) to viral vectors are a major hurdle to using these advanced biologics as traditional drugs. NAbs block therapeutic gene delivery in those with pre-existing vector immunity, and prevent vector dose titration after the first treatment. Of the strategies in development to reduce the effect of pre-existing NAbs or blunt the generation of antibodies during treatment, most suffer from limited efficacy to bring NAb titers below a level that permits vector administration, and none demonstrate instantaneous NAb suppression. Previously we demonstrated that a soluble fragment of mycoplasma Protein M transiently blocks NAbs to adeno-associated virus (AAV), which enabled successful in vivo systemic gene delivery over a 1,000-fold concentration range of antiAAV NAbs as compared to controls without Protein M where the same AAV dose was neutralized. Protein M binds to the Fab region of all antibody classes at a conserved site common to humans, non-human primates, rodents, and most mammalian species. Protein M binding temporarily blocks all antigen recognition through steric hinderance of the complementarity-determining regions, and generates a transient gene delivery window whereby any vector is universally protected from neutralization. Additionally, we demonstrate that use of Protein M with a variety of vectors instantaneously blocks NAbs for successful gene delivery, enabling robust gene expression in the presence of neutralizing serum. While generating feasibility data we quickly discovered that Protein M is highly unstable at body temperature, posing a significant challenge for in vivo use and scalable protein manufacturing. Instantaneous Protein M unfolding occurs at 41°C, resulting in precipitation and aggregation. Incubating a truncated naturally occurring Protein M sequence at 37°C caused protein

unfolding after only 15 minutes and loss of antibody blocking function. Therefore, we used structural and computational modeling to design 850+ Protein M analogs with enhanced properties. We produced a library of stabilized mutants using *in silico* site saturation mutagenesis and free energy prediction, and then screened single and combinatorial mutants. Stabilizing mutations resulted in a distribution of analogs with increased melting temperatures, from 42°C degrees to 65°C and greater. Improvements in thermostability were accompanied by significantly decreased aggregation, increased solubility, and ability to highly concentrate the protein. Stabilized Protein M variants improved in vitro function, whereby several analogs retained antibody blocking

ability after more than 72hrs at 37°C. Using an engineered analog, PM129, we now demonstrate successful readministration of AAV in mice immunized by AAV one-month before secondary administration. PM129 blocks NAbs after systemic intravenous dosing shortly before AAV administration, as well as when PM129 is admixed with AAV and administered in a single intramuscular injection. While PM129 is a novel foreign protein with the capacity to elicit inhibitory antibodies to itself, we are able to successfully redose PM129 and AAV even when prior doses of PM129 have been administered. This suggests PM129 is capable of outcompeting self-neutralizing antibodies, which likely represent only a small fraction of the total serum antibody pool. Finally, safety assessments of PM129 were conducted in mice demonstrating no observed adverse reactions in over 40 mice included in the high dose cohort. Minimal B cell or T cell proliferation was observed after in vivo administration and collected serum samples failed to activate complement C5 or demonstrate immune complex formation.

177. *In Vivo* HSC Gene Therapy with High-Level, Erythroid-Specific Expression of a Secreted SARS-CoV-2 Decoy Receptor

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We developed a technically simple and portable *in vivo* hematopoietic stem cell (HSC) transduction approach that involves HSC mobilization from the bone marrow into the peripheral blood stream and the intravenous injection of an integrating, helper-dependent adenovirus (HDAd5/35++) vector system. HDAd5/35++ vectors target human CD46, a receptor that is abundantly expressed on primitive HSCs. Transgene integration is achieved by a hyperactive Sleeping Beauty transposase (SB100x) and transgene marking in peripheral blood cells can be increased by *in vivo* selection. Here we directed transgene expression to HSC-derived erythroid cells using beta-globin regulatory elements. We hypothesized that the abundance and systemic distribution of erythroid cells can be harnessed for high-level production of secreted therapeutic proteins. We first demonstrated that our approach allowed for sustained, erythroid-lineage expression of a bioengineered human factor VIII, termed ET3, leading to phenotypic correction of the bleeding defect in $hCD46^{+/+}/F8^{-/-}$ hemophilia A mice as measured by chromogenic assay, aPTT assays and tail bleeding assays (PMID: 31585952). We then used this approach for continuous expression of a SARS-CoV-2 decoy receptor, i.e. a secreted, human ACE2 extracellular domain fused to human constant IgG1 domains to form an antibody-like structure (sACE2-Ig) capable of mediating virus opsonization. We performed *in vivo* HSC transduction of CD46-transgenic mice with a HDAd5/35++-sACE2-Ig vector. Serum sACE2-Ig levels reached 500-1300 ng/ml after *in vivo* selection. The sACE2-Ig protein in the serum from these animals was active in blocking the infection of $293\text{-}ACE2^{+}$ cells with a CoV2-Spike proteinpseudotyped lentivirus vector. Lineage-negative bone marrow cells from HDAd5/35++-sACE2-Ig-transduced CD46-transgenic mice will be transplanted into lethally irradiated K18-hACE2 transgenic

mice. The resulting mice will express sACE2-Ig and will be susceptible to SARS-CoV-2 infection. Furthermore, we performed *in vivo* HSC transduction with the integrating HDAd5/35++sACE2-Ig vector in a rhesus macaque. HSC mobilization by subcutaneous administration of G-CSF/plerixafor and intravenous HDAd injection at dose of $3.2x10^{12}$ vp/kg was well tolerated after appropriate cytokine prophylaxis (dexamethasone, tocilizumab, anakinra). The HSC transduction efficacy and serum sACE2-Ig levels after *in vivo* selection will be reported. Ultimately, we plan to test whether continuous sACE2-Ig expression could provide protection against challenges with CoV-2 variants in the mouse and NHP models.

178. A Platform for Genome Editing of Human B Cells to Produce Single-Chain Antibody-Like Molecules That Recapitulate Antibody Functionality

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Genome editing of B cells offers opportunities to create cells that express antibodies with characteristics that cannot be elicited by vaccination. However, the complex regulatory pathways governing antibody function, which include alternative splicing, class switch recombination, and somatic hypermutation, suggest that editing the endogenous immunoglobulin (Ig) locus may be required to fully reconstitute such functions in an engineered antibody. Towards that goal we have developed a strategy to engineer the human Ig locus based on the design of single-domain antibodies (sdAbs) - heavy-chain only antibodies found in camelids that comprise an antigen-binding VHH domain and heavy chain Fc region. By inserting a promoterVHH-splice donor cassette within an intron of the human IgG1 gene (*IGHG1*), a spliced mRNA is produced that fuses the VHH to constant domains of IgG1 and results in both a recombinant membrane-bound B cell receptor (BCR) and the matched secreted

antibody. As a proof of principle, we used CRISPR/Cas9 to engineer human B cell lines with HIV Env-specific VHH domains. We observed anti-HIV BCR cell surface expression, signaling after engagement with the HIV Env antigen, and secretion of functional sdAbs capable of neutralizing HIV. Long-term culture of the cells resulted in signatures of somatic hypermutation, with the sequence changes strongly localized to AID hotspot motifs, and consequential alterations in antibody functionality. Such an ability may provide advantages against a highly mutable target such as HIV. Expanding the approach to primary human B cells, we combined CRISPR/Cas9 with AAV6 homology donors to insert anti-HIV VHH cassettes at *IGHG1*. Over 20% of cells expressed HIVspecific human IgG as measured by flow cytometry, and the engineered B cells could be expanded >50-fold over 11 days using an animalcomponent free system. After *in vitro* differentiation that skewed the B cells towards an antibody-secreting cell phenotype, we observed a shift in splicing of the chimeric antibody mRNA towards the secreted isoform and increased anti-HIV sdAb secretion. Importantly, antiHIV sdAb secretion tracked with total IgG secretion by unmodified cells on a per cell basis (R^2=0.99), indicating that alternative splicing of the transgene was co-regulated with endogenous antibody splicing by the differentiation state of the B cell. Finally, supernatants from engineered B cells were able to neutralize HIV infection with an IC50 equivalent to the recombinant VHH-Fc protein, indicating that sdAbs generated by this strategy are properly produced and secreted despite the presence of other endogenous IgGs in this system. In conclusion, we have established a versatile platform to reprogram human B cells to express engineered single-chain antibodies. It can be used with a variety of antigen-recognizing domains including VHH domains, scFvs and soluble receptor derivatives, expanding the range of antibodylike molecules that can be produced from the Ig locus. Multiplexed cassettes, comprising for example multiple VHH domains linked together by flexible linkers, can also be inserted and used to produce molecules with enhanced or multivalent functionalities. We believe the technology is broadly applicable and could be considered for any antibody-based therapy that would benefit from long-term *in vivo* production, including targets in cancer, autoimmune diseases, or other infectious diseases.

179. Immune Modulation Preceding AAV9GLB1 Gene Therapy Preserves the Possibility for Re-Dosing in Children with GM1 Gangliosidosis

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GM1 gangliosidosis is a rare, autosomal recessive, multisystem lysosomal storage disorder with relentless neurodegeneration and no effective therapy. AAV9 has broad distribution with intravenous administration and demonstrated tropism to the CNS. However, preexisting immunity to AAV9 decreases the effectiveness of transgene delivery and precludes the re-dosing of children as they grow. Here we present results of 6 Type II GM1 patients (ages 2.5 to 9 years) who received 1.5 x 10^{15} vg/kg AAV9-*GLB1* intravenously following a novel immune modulation pre-conditioning regimen to deplete CD19+ B cells and inactivate CD3+ T cells. All 6 patients received 4 intravenous doses of rituximab (375 mg/m^2) beginning 21 days prior to gene transfer and oral rapamycin (0.5-1 mg/m^2/day) to maintain a trough level of 5-10 ng/mL beginning 21 days prior to gene transfer and continuing 180 days following gene delivery. Methylprednisolone (1 mg/kg IV) was administered 60 minutes prior to gene transfer and prednisone (0.5mg/kg/d) was given orally for 3 days following dosing. CD19+ B cells were depleted following 2 does of rituximab in all 6 subjects with recovery beginning 3-6 months following gene delivery. There were no serious infections in any subject with the exception of one patient who developed bacterial sepsis from PICC line malfunction at 2 weeks following gene transfer. Quantitative immunoglobulins remained within normal ranges for all subjects through 6 months following gene transfer. Side effects of mucositis (4 patients) and mild neutropenia (3 patients) resolved without sequalae. IgM and IgG antibody titers to AAV9 did not change in 4 of 6 subjects. One subject, thought to be possibly pre-immune to AAV, had a delayed IgG response beginning at 14 days following vector administration that remained elevated through 180 days. A second subject had delayed IgM response to viral capsid that peaked at 30 days followed by a modest IgG response beginning at 30 days and persisted through 180 days. The pharmacokinetics of vector genome copies in serum for these 2 subjects were not different than the remaining 4 subjects who did not show a response. Following gene transfer, liver functions including alanine aminotransferase (ALT) and gamma-glutamyl-transferase (GGT) were within normal limits. Baseline aspartate aminotransferase (AST) was mildly elevated in all subjects consistent with our observations in a cohort of GM1 patients (mean=61.35 U/L, N=38). No subject had AST elevations greater than 3X their baseline value. C3 and C4 remained within the normal range and 3 subjects had mild thrombocytopenia (lowest value of 88 K/ mcL). Immune modulation was safe and well tolerated in our cohort and there were no complications in the days immediately following gene delivery. Two patients developed delayed antibody to the viral capsid, one of whom was likely pre-immune to AAV. Four patients showed no rise in antibody titer following gene transfer leaving open the possibility for re-dosing with the same AAV9 vector in the future.

New Gene Editing Technologies and Applications

180. Modulation of DNA Repair Pathways by HDR-CRISPR Promotes Seamless Genome Editing in Primary Human Hematopoietic Cells

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The CRISPR-Cas system is a robust platform for genome editing. The introduction of a DNA double-strand break (DSB) at precise gene locations can be exploited to achieve targeted gene knockout by harnessing the error-prone non-homologous end-joining (NHEJ) pathway. However, using CRISPR-Cas technology for precise genome editing via homology-directed repair (HDR) remains challenging, with HDR frequencies below the threshold required for clinical translation. Common strategies to increase HDR-mediated DSB repair include the use of chemicals either to inhibit NHEJ or to arrest the cells in those cell cycle phases when HDR is most active. However, the global effects of these drugs pose serious safety concerns if applied in clinically relevant settings. To address this issue, we devised a strategy to recruit HDR-promoting factors or NHEJ-inhibiting proteins to the DSB site. This is achieved via the direct fusion of particular protein-protein interaction domains to the Cas9 nuclease. We generated 16 different Cas9-fusion proteins (referred to as HDR-CRISPR) and extensively investigated their impact on DNA repair pathway choice by using two reporter systems, the traffic light reporter (TLR) and the BFPto-GFP (B2G) assay. These two assays allowed us to investigate the outcome of DNA repair mediated by a DNA donor supplied either as a plasmid or oligodeoxynucleotides (ODN) respectively. Our results indicate that HDR-CRISPRs enhanced HDR frequency 3-fold over baseline levels. The simultaneous reduction of NHEJ-mediated repair

resulted in a 5-fold increase in the HDR:NHEJ ratio when using our best performing HDR-CRISPR. Next, we assessed the capability of HDR-CRISPR to precisely integrate a large GFP expression cassette into the endogenous *AAVS1* safe harbor locus of K562 and Jurkat cell lines. Independently of the cell type, the use of HDR-CRISPR resulted in a 2.5-fold increase in targeted integration as compared to samples receiving the unmodified Cas9. The most efficient HDR-CRISPR fusion was then tested for its ability to promote HDR-mediated repair in clinically relevant primary human cells. HDR-CRISPR was delivered to T lymphocytes and hematopoietic stem cells (HSCs) in the form of RNA. Using an appropriate ODN as a repair template, we aimed at introducing a stop codon within exon 3 of the *CCR5* gene to generate immune cells resistant to HIV infection. HDR-CRISPR led to a 2-fold increase in precise genome editing events as compared to the use of an unmodified Cas9. In conclusion, our data support the hypothesis that DSB repair choice can be altered through the local recruitment of key DNA repair factors capable of either promoting HDR or inhibit NHEJ, also in clinically relevant cells.

181. Capturing and Characterizing Single Cell Allelic Heterogeneity of CRISPR-Cas9 Gene Editing *In Vivo*

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Translating CRISPR/Cas9 technology into *in vivo* therapeutic applications necessitates a strong understanding of the potential sites of delivery and the nature of subsequent gene editing events. Editingdependent reporter mouse strains (e.g. Ai6 and Ai14 (both containing lox-stop-lox-fluorescent protein at the *ROSA26* locus)) in combination with adeno-associated virus (AAV) delivered CRISPR/Cas9 offers a sensitive way to address some of these delivery and editing questions. In some occurrences, a deletion affecting both alleles of a gene may be desired. To assess the frequency and fidelity of biallelic editing, we generated compound Ai14/Ai6 heterozygote mice. Injection of AAVs encoding Cas9 protein and gRNAs targeted to the transgenic *ROSA26* locus will edit the stop cassette and activate tdTomato and/or ZsGreen from the Ai14 and Ai6 reporter alleles, respectively. Interestingly, after AAVgRNA delivery, the most frequent outcome was that only one of the two reporter alleles was activated in transduced hepatocytes, suggesting allelic heterogeneity within single cells following *in vivo* editing. The experiment was repeated *in vitro* using Ai14/Ai6 mouse embryonic fibroblasts and SpCas9/gRNAs- or SaCas9/gRNAs-expressing plasmids. Following transfection, we again observed heterogeneity in reporter activation. Next, we used flow to separate cells into double positive (tdTomato+/ZsGreen+), single positive (tdTomato+/ZsGreen- or tdTomato-/ZsGreen+), or double negative (tdTomato-/ZsGreen) populations and sequenced DNA from tdTomato-/ZsGreen+ single positive cells at the Ai14 (non-expressing) and Ai6 (expressing) alleles. As expected, the Ai6 allele contained the desired CRISPR-mediated deletion of the stop cassette. However, at the non-expressing Ai14 allele, the most common editing outcome was indels at both gRNA cuts sites without deletion of the stop cassette. Contrary to our expectation, the apparent reason for non-activation at the Ai14 allele was not lack of editing, but rather, undesired editing. The NHEJ DNA-repair inhibitor,

Ku57788, increases CRISPR deletion efficiency *in vitro*. To test its effects on biallelic deletions, Ai14 /Ai6 mouse embryonic fibroblasts were treated with Ku57788 and transfected with SpCas9/gRNAs- or SaCas9/gRNAs-expressing plasmids. Flow-sorted cells exhibited a marked increase in single positive cells of both reporter alleles, but most importantly exhibited a near 2-fold increase in the number of cells with a successful biallelic deletion. This suggests that efforts to transiently inhibit DNA repair may increase biallelic CRISPR deletion efficiency.

182. Dual-HDR Editing Strategies for the Development of Islet-Specific Regulatory T Cells (EngTregs) for Restoration of Immune Tolerance in Type 1 Diabetes

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Adoptive transfer of engineered regulatory T cells (EngTregs) represents an emerging therapeutic approach to promote immune tolerance in the setting of stem cell or solid organ transplantation,

and in autoimmune diseases including type 1 diabetes (T1D). We previously developed robust methods to generate polyclonal EngTregs from CD4+ T cells by homology-directed-repair (HDR)-based gene editing. This approach introduces a ubiquitous promoter (MND) into the *FOXP3* locus downstream of the Treg-specific demethylated region (TSDR), resulting in high level FOXP3 expression and stable Treg-like phenotype and function. Polyclonal EngTregs cells generated by this approach are beneficial for systemic autoimmune conditions (including IPEX and GvHD) but lack the fine-specificity required to control tissuespecific autoimmune diseases driven by locally-expressed self-antigens. In this study, we describe a robust dual-HDR approach to generate and enrich antigen-specific EngTregs cells. Knock-out of the endogenous T cell receptor alpha constant (*TRAC*) gene and replacement with an islet-specific TCR is predicted to generate EngTregs capable of localizing to APCs expressing islet antigens in the pancreatic lymph nodes and/or pancreas. We evaluated three strategies for introducing an islet-specific TCR into the *TRAC* locus in parallel with HDR-editing of the *FOXP3* locus. To overcome the anticipated lower efficiencies of dual-HDR editing, we generated HDR donor cassettes designed to simultaneously introduce a heterodimeric, chemically-induced signaling complex (CISC) that mimics IL-2 signaling in response to an exogenous dimerizer, rapamycin. For our dual-HDR approach, the dimerizing components of the CISC cassette were introduced via two separate locus-specific editing events (in *TRAC* and *FOXP3*), thereby enabling specific selection and enrichment of only the dual-edited, islet-specific EngTregs. We first attempted a *TRAC* knock-in cassette incorporating an MND promoter, the full islet-TCR coding domain, and half of the CISC cassette. This strategy produced dual-edited cells that enriched in response to CISC dimerization. However, due to the large size of the TRAC-targeting repair template, the initial editing rates were low. To reduce the size of the HDR cassette, we pursued a promoter capture strategy, introducing the islet TCR downstream of the endogenous TRAC promoter. Notably, split-CISC test constructs using fluorophore markers showed that the these dual-edited cells failed to enrich, implying that CISC functionality requires high surface density of the synthetic receptor. Taking these findings into account, we designed an in-frame knock-in construct with an MND promoter driving CISC expression followed by the partial islet-TCR sequence (bearing the TCR beta chain and the alpha chain variable region) hijacking the endogenous alpha constant gene. Using this approach, we observed strong CISC-based enrichment of dual positive, antigen specific, EngTreg cells. After culturing cells in dimerizer, edited cells enriched from initial dual HDR-editing rates of 5-10% to >80% within 7 days, and these populations expressed high-levels of FOXP3 and relevant Treg surface markers. Our findings demonstrate that dualHDR editing using a 'split IL-2 CISC' is a viable strategy for generating antigen-specific EngTreg cells. This approach has the capacity to deliver targeted immune tolerizing therapy to treat or prevent T1D and is likely to be easily adapted for use with alternative TCRs (or CARs) designed to target tissue-specific autoimmune or inflammatory diseases.

183. A Dimeric, Luminescent Biosensor for Imaging Unique DNA Sequences in Individual Cells

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One of the most prominent bottlenecks in the gene editing process is the ability to identify and isolate individual cells with desired edits within a population of treated cells. Current approaches typically require time-consuming and labor-intensive single cell isolation and population expansion followed by destruction of some portion of an expanded cell population for downstream *in vitro* analysis of DNA sequence content. Cell types that exhibit low efficiencies in transfection, editing, single cell isolation, or population expansion can be particularly challenging. To compound this problem, homology directed repair (HDR) can exhibit extremely low efficiency in certain cell types. A promising alternative to these and other destructive DNA detection assays could be the direct biosensing of edited DNA sequences in living cells. In recent years, an extensive arsenal of biosensing tools has been developed based on the clustered regularly interspaced short palindromic repeat (CRISPR) platform, including those that detect the presence of specific DNA sequences both *in vitro* and in live cells. In fact, the CRISPR/Cas gene editing system has been extensively modified for imaging endogenous genomic loci, but the vast majority of current approaches utilize monomeric fluorescent reporter-based biosensors, such as dCas9-GFP. A major drawback to these systems is that each monomeric sensor produces a signal whether bound to its target DNA or not, resulting in a high fluorescent background that negatively impacts the signal-to-noise ratio. For this reason, such "always-on" sensors must rely on obtaining a high local concentration of probes to distinguish signal from noise, limiting their use to highly repetitive elements that can be targeted by one gRNA or to unique sequences targeted by 50 or more gRNAs. In contrast, dimeric "turn-on" DNA biosensors offer the possibility of achieving signal production solely upon binding of two subunits to the target DNA and reassembly of a bright reporter. Furthermore, luminescent reporters are an attractive alternative to fluorescent reporters in biosensing experiments as cellular background luminescent signal is essentially nonexistent. This is due to the different nature of light production in luminescent reporters where a catalytic reaction of an enzyme with its substrate produces light, eliminating the need for exogenous excitation light.Taking these points into consideration, we have developed a luminescence-based, dimeric DNA sequence biosensor that provides a sensitive readout for DNA sequences through proximity-mediated reassembly of two independently optimized fragments of NanoLuc luciferase (NLuc), a small, bright reporter. Reconstitution of NLuc becomes more favorable upon binding of two guide RNAs (gRNAs) to two DNA target sites with a defined orientation and spacing. Using this "turn-on" probe, we demonstrate rapid and sensitive detection of as low as 190 amol transfected target DNA, presenting a reliable approach for DNA biosensing. Across several cell-based delivery approaches, we were able to achieve approximately 2.5 - 20-fold increase in signal in live populations of cells transfected with the dimeric biosensor and various target DNA scaffolds compared to populations transfected with the dimeric biosensor but no target DNA. The future goals for this system include detection of single-copy genomic sequences at endogenous loci and validation of gene sequence changes after genome editing experiments in live cells.

184. Efficient CRISPR-Cas9-Mediated Gene Knockout and Interallelic Gene Conversion in Human Induced Pluripotent Stem Cells Using Non-Integrative Bacteriophage-Chimeric Retrovirus-Like Particles

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The combination of CRISPR/Cas9 technology with human induced pluripotent stem cells (hiPSC) has tremendous potential for basic research and cell-based gene therapy. However, fulfilling these promises relies on our capacity to efficiently deliver exogenous nucleic acids into these cells and harness the repair mechanisms induced by the nuclease activity.Since gene editing systems require low and shortterm expression in order to avoid off-target effects, RNA delivery is favored over DNA delivery. RNA delivery presents major advantages compared to DNA delivery, being that it is completely safe and devoid of any recombination events in the host genome. It is actually the most versatile, flexible, and safe mean for human therapy.Here, we investigated the potential of bacteriophage-chimeric retrovirus-like particles for the non-integrative delivery of RNA molecules in hiPSC. We found that these particles efficiently convey RNA molecules for transient expression in hiPSC, with minimal toxicity and without affecting cell pluripotency and subsequent differentiation. We then used this system to transiently deliver the CRISPR-Cas9 components (Cas9 mRNA and sgRNA) to generate a gene knockout with a high indel level (up to 85%) at several loci into hiPSC. Strikingly, when using an allele-specific sgRNA at a locus harboring compound heterozygous mutations, the targeted allele was not altered by NHEJ/MMEJ, but was repaired at high frequency using the homologous wild type allele, suggesting interallelic gene conversion.Our results highlight

the potential of bacteriophage-chimeric retrovirus-like particles to efficiently deliver RNA molecules in hiPSC, and describe for the first time genome engineering by gene conversion in hiPSC. Harnessing this DNA repair mechanism could facilitate the therapeutic correction of human genetic disorders in hiPSC.

185. Triggering P53 Activation and Trapping of Transcriptionally Active Recombinant AAV Sequences Are Inadvertent Consequences of HSC Genome Editing

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Gene editing by homology-directed repair (HDR) in human hematopoietic stem cells (HSC) holds therapeutic potential for the treatment of hematological diseases by *in situ* correction of pathogenic mutations or site-specific integration of a transgene expression cassette. Induction of DNA double strand breaks by nucleases (e.g. CRISPR/
Cas ribonucleoprotein) and cell transduction with single-stranded Adeno-Associated Vector serotype 6 (ssAAV6) to deliver the donor template for HDR cumulativelytrigger a robust p53-dependent DNA damage response (DDR), which delays HSC proliferation, lower the yield of edited cells and ultimately results in oligoclonal reconstitution after xenotransplantation. Transient p53 inhibition released cell cycle block and improved size and clonal composition of the human graft. Although ssAAV6 is the major contributor to p53 pathway activation, the elements responsible for such DDR signaling as well as their persistency in edited HSC have not been fully characterized. We did not observe significant differences in kinetics or extent of p53 pathway activation due to targeted loci, transgene cassette or ssAAV6 purification process, suggesting a shared viral component as DDR trigger. However, HSC edited and transduced with genomefree ("empty") AAV6 did not promote p53 response over nucleaseonly treated cells and robustly engrafted in immunodeficient mice, thus ruling out capsid-dependent effect and confirming that ssAAV genomes can activate the p53 pathway. Delivery of the HDR template by self-complementary (sc)AAV6 triggered a more prolonged p53 transcriptional response and further reduced repopulation capacity of edited cells compared to ssAAV6-edited ones. Since recombinant (r) AAV genomic elements may trigger detrimental cellular responses in cultured HSC, we evaluated persistence of vector sequences in edited cells by deep sequencing the edited allele. A low fraction of HSCs unexpectedly carried on-target trapping of rAAV DNA fragments despite the transient delivery of editing components in these actively proliferating target cells. This finding was consistent across different editing loci and transgene cassettes and was confirmed in other human primary hematopoietic cells. Edited HSC with on-target trapping of rAAV DNA fragments engrafted in immunodeficient mice and

persisted long-term after transplant (median allele frequency < 0.5%). Sequence alignment of trapped fragments to the parental rAAV genome showed prevalence of the inverted terminal repeats (ITR) including the Rep binding element and the terminal resolution site. Of note, hematopoietic cells transduced with ssAAV6 harboring a promoterless reporter cassette showed low but detectable ITR-dependent transcriptional activity. We are currently investigating the underlying mechanism of such rAAV fragment trapping and whether